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Relief of polarity caused by transposon Tn*5*: application in mapping a cloned region of the *Escherichia coli uvrB* locus essential for UV resistance

(Recombinant DNA; transposon Tn*1*; cloning in plasmid)

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SUMMARY

The structure and function of recombinant plasmid pNP5, which consists of vector pMB9 and a 2.5 kb *Eco*RI fragment harbouring the *Escherichia coli uvrB* gene, has been investigated. Insertional inactivation with the transposons Tn*1* (Ap^r) or Tn*5* (Km^r) has been used to determine the region on pNP5 DNA that is essential for UV resistance in *uvrB* deletion strains. This region spans approx. 1.8 kb and is separated by at least 280 bp from the pMB9 promoter to which it has been fused.

Furthermore, a procedure is described to eliminate the polarity exerted by the transposon Tn*5*. A combination of in vitro digestion of pNP5::Tn*5* DNA with restriction endonuclease *Xho*I, followed by ligation and subsequent in vivo propagation of the resulting plasmid DNA yields predominantly pNP5 molecules with a site-specific non-polar mutation. The method allows an investigation of cloned complex genetic units, such as operons.

INTRODUCTION

Insertional inactivation with transposons has been a useful technique to determine the location of genes on plasmids. Integration of a transposon into a plasmid frequently results in the loss of a function which is accompanied by some phenotypical alteration. The position of a transposon on a plasmid can be determined either by heteroduplex mapping using electron microscopy or by restriction enzyme analysis of the DNA. Several functions of the plasmids ColE1 and CloDF13 have been mapped successfully, employing various transposons carrying antibiotic resistance determinants as easily selectable markers (Dougan et al., 1978; Inselberg, 1977; Van Embden et al., 1978).

We have employed the technique of insertional inactivation, using transposons Tn*1* and Tn*5* coding for, respectively, Ap^r and Km^r, to determine the position of the cloned *E. coli uvrB* locus on a recombinant plasmid, denoted pNP5 (Pannekoek et al., 1978). Plasmid pNP5 consists of the vector pMB9 and an *Eco*RI fragment, harbouring the *uvrB* locus, which has been inserted into the unique *Eco*RI site of the vector. In this study it was assumed that insertion of

Abbreviations: Ap^r, Ap^s, ampicillin resistance and sensitivity, respectively; bp, base pair; IR, inverted repeat; kb, kilobase pair; Km^r, Km^s, kanamycin resistance and sensitivity, respectively; Tc^r, Tc^s, tetracycline resistance and sensitivity, respectively.

a transposon in the cloned *uvrB* locus results in the loss of UV resistance which is displayed by *uvrB* deletion strains carrying plasmid pNP5. Previously, we have shown that the expression of the cloned *uvrB* locus is mediated by an effective promoter located on the vector pMB9 (Pannekoek et al., 1979). Moreover, DNA sequencing of a 354 bp *Eco*RI-*Hin*dIII fragment of pMB9 located adjacent to the cloned *uvrB* locus has confirmed the presence of a promoter which can initiate transcription of the *uvrB* locus (Pannekoek et al., 1980). Clearly, although the genuine *uvrB* transcriptional regulatory elements are lacking, plasmid pNP5 contains the genetic information to fully complement chromosomal *uvrB* mutations. To obtain further information on the genetic organization of the *uvrB* locus we have investigated which part of the cloned *Eco*RI fragment is required for UV resistance and which part(s) can be altered without loss of UV resistance in *uvrB* deletion strains.

Alteration of a particular phenotype due to insertion of a transposon and a subsequent mapping of the position of the transposon on the plasmid may, however, be misinterpreted. (i) Insertion of a transposon in a segment of a gene which does not contribute to the phenotype examined erroneously may lead to the assumption that the particular segment will not be part of that gene. (ii) Transposons are either polar and mutagenic in one orientation and just mutagenic in the opposite orientation (e.g. Tn*1*) or they are polar and mutagenic in both orientations (e.g. Tn*5*) (for a review see Kleckner, 1977). Consequently if a gene is part of an operon then insertion of a transposon in the operon at a site ahead of that gene might lead to inactivation due to a polar effect. One may assess whether or not the gene in question is part of an operon by relieving the polar effect of the transposon. In the case of Tn*1*, but not of Tn*5*, this can be achieved by inversion of the orientation of the transposon. In this paper we describe a new procedure to eliminate the polar effect of Tn*5*. The method, which involves the removal in vitro of internal fragments of the transposon and subsequent in vivo propagation of the resulting products, yields mainly nonpolar mutations. The potency of a general application of this procedure will be discussed.

MATERIALS AND METHODS

(a) Bacteria and bacteriophages

The following strains of *E. coli* K-12 were used: LBE1339 containing the R-factor RP4 (from P. Hooykaas); HP3435 *sup*-6 Δ(*bioFCD-uvrB-chlA*) and HP3484 prototrophic and lysogenic for λ*b*221*rex*::Tn*5c*I857 are from our laboratory. All strains are F^-. Phage λ*b*221*rex*::Tn*5c*I857 was obtained from D.E. Berg.

(b) Transposition

Transposition of Tn*1* (Ap^r) into plasmid pNP5 was carried out as follows. Strain LBE1339 harbouring RP4 ($Ap^rTc^rKm^r$) was transformed with pNP5 DNA ($Tc^rcol^{imm}Uv^r$) and col^{imm} transformants were selected (Dougan and Sherratt, 1977). Bacteria with the desired phenotype were grown in L-broth containing the appropriate antibiotics for 40 generations at 32°C (Kretschmer and Cohen, 1977) and subsequently, plasmid DNA was isolated (Meagher et al., 1977). 50 μg of the resulting plasmid DNA preparation was used to transform strain HP3435(Δ*uvrB*) and clones were selected having an $Ap^rTc^rcol^{imm}$ (Km^s) phenotype. The Uv^r phenotype of the resulting clones was tested as outlined previously (Pannekoek et al., 1979).

Integration of Tn*5* (Km^r) into pNP5 was done employing the following procedure. Strain HP3484 lysogenic for phage λ*b*221*rex*::Tn*5c*I857 was transformed with pNP5 DNA, selecting for Tc^r clones. Transformed bacteria were grown as mentioned above and plasmid DNA was isolated. Again, 50 μg of plasmid DNA was used to transform strain HP3435 in order to select Tc^rKm^r bacteria. Finally, these clones were tested for their resistance against irradiation with UV light.

(c) Analysis of DNA with restriction endonucleases

Incubations of plasmid DNA (50 μg/ml) with restriction endonucleases were carried out for 1 h at 37°C with 2–3 units of enzyme/μg of DNA. The buffers used for various restriction endonucleases were made according to the instructions of the manufacturers. Digestions were arrested by heating for 5 min at 65°C, followed by quenching in ice. Samples

to be analyzed were made 3 to 5% (v/v) Ficoll and submitted directly to electrophoresis on 0.8% agarose (Helling et al., 1974) or on 5% polyacrylamide slab gels (Bolivar et al., 1977).

(d) Formation of covalently closed monomers

2 μg of *Xho*I digested DNA was incubated in a volume of 1 ml for 16 h at 14°C using the following conditions: 50 mM Tris · HCl (pH 7.5), 10 mM $MgCl_2$, 0.2 mM ATP, 10 mM dithiothreitol, 0.1 mM EDTA and 0.5 unit of T4-DNA ligase. The mixture was extracted with phenol and chloroform, precipitated with two volumes of ethanol and then dialyzed against 5 mM Tris · HCl (pH 7.2), 0.1 mM EDTA. The frequency of transformation with this ligated DNA preparation was about 10^{-4} transformants per viable bacterium.

(e) Enzymes

The restriction endonucleases *Eco*RI, *Bam*HI, *Hin*dIII and the enzyme T4-DNA ligase were from Miles Laboratories Inc. (Elkhart, IN). *Bgl*I and *Xho*I were purchased from Biolabs (Beverly, MA), whereas *Pst*I, *Hin*dII were from Boehringer (Mannheim, F.R.G.). The enzymes *Sal*I, *Bst*EII, *Alu*I and *Hae*III were kindly donated by H.L. Heyneker (Genentech Inc., San Francisco, CA) and *Hin*fI was a gift from J. Maat (Physiological Chemistry, Leiden, The Netherlands).

RESULTS

(a) Integration of transposons into plasmid pNP5

Plasmid pNP5 has been constructed by introduction of an *Eco*RI fragment (2.5 kb), derived from a *uvrB*-transducing phage λ*b2att*^{2} (λ*b2int*am6(Δ*bioAB*) *bioFCD*$^+$*uvrB*$^+$*c*I857), into the unique *Eco*RI site of plasmid pMB9 (5.3 kb), which carries genes specifying Tc^r and colicin immunity (col^{Imm}) (Rodriguez et al., 1976; Pannekoek et al., 1978). The cloned *Eco*RI fragment contains, besides the *uvrB* locus, a segment of DNA, located between 68.3% and 75.4% of the pNP5 map, which originates from the opposite side of the λ attachment site on the chromosome as the *uvrB* locus (Fig. 1). Presumably, this region has no function in the Uv^r phenotype displayed by *uvrB* strains harbouring pNP5. The region of our interest is thus located between 75.4% and 100% and comprises approx. 1920 bp.

The R-factor RP4 was employed as a source for the transposable element Tn*1* (Ap^r). RP4 carries determinants for resistance against the antibiotics ampicillin, tetracycline and kanamycin; transposition has been demonstrated for Ap^r only (Hedges and Jacob, 1974). Integration of Tn*1* into plasmid pNP5 was accomplished by transforming an RP4-containing *E. coli* strain with pNP5 DNA, followed by subsequent culturing of the transformed strain at 32°C. DNA was prepared from this strain after amplification with chloramphenicol. The preparation will contain mainly pNP5 DNA, but also a minor fraction of pNP5 containing Tn*1* (pNP5::Tn*1*) and non-amplified RP4

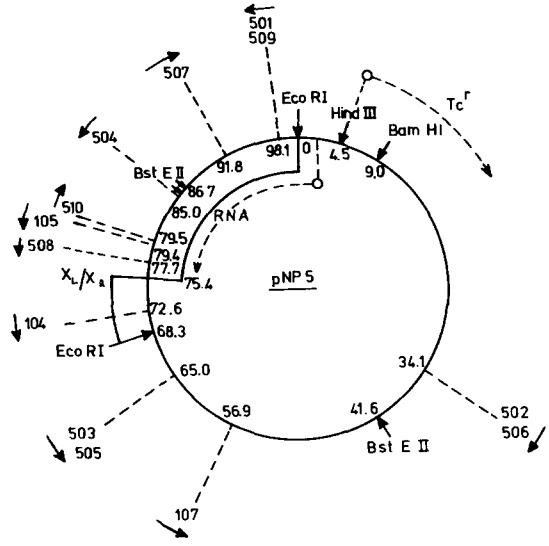

Fig. 1. Diagrammatic representation of the structure of plasmid pNP5. Restriction sites for the enzymes *Eco*RI, *Hin*dIII, *Bam*HI and *Bst*EII are given in percentages of the length of pNP5 DNA (7.8 kb). The region from 68.3% to 75.4% has been derived from the *gal* side of the lambda attachment site on the chromosome and the region from 75.4% to 100% originates from the *bio-uvrB* side. Integration sites of the transposons Tn*1* and Tn*5* are indicated; insertion sites designations (three-digit numbers) starting with a "5" are derived from Tn*5*, those with a "1" from Tn*1*. The solid arrows represent the direction of transcription of, respectively, the Ap^r-gene (Tn*1*) and the Km^r-gene (Tn*5*). The direction of transcription of the *uvrB* gene and the Tc^r determinant are indicated by the dotted arrows (Pannekoek et al., 1979).

DNA. The mixture was used to transform strain HP3435 *uvrB* and transformants were selected for a $Ap^rTc^rcol^{imm}$ (Km^s) phenotype. The majority (about 90%) of these clones were still resistant to UV-irradiation, suggesting that Tn*1* is preferentially inserted into the vector part of pNP5. Analysis of the DNA of $Ap^rTc^rcol^{imm}Uv^r$ clones showed that most of the insertions had occurred at 56.9% of the pNP5 map (Fig. 1). However, we found one Uv^r clone containing pNP5::Tn*1* DNA with the transposon inserted within the cloned *Eco*RI fragment. Analysis with restriction endonucleases of the DNA (pNP5::Tn*1* No. 104) demonstrated that the insertion had occurred within the segment between 68.3% and 75.4%. We have argued before that this segment is not related to the *uvrB* locus. Hence, this result confirms our previous conclusion on the composition of the cloned *Eco*RI fragment (Pannekoek et al., 1978).

Transformants which displayed a $Ap^rTc^rcol^{imm}Uv^s$ phenotype were further examined. DNA prepared from several independent clones appeared to be composed of identical pNP::Tn*1* (No. 105) molecules. Clearly, integration of Tn*1* into the *uvrB* locus had occurred at a single site. Plasmid pNP5 contains a unique restriction site for the enzyme *Hin*dIII (4.5%) and no site for *Pst*I. On the other hand, transposon Tn*1* has no site for *Hin*dIII and harbours three sites for *Pst*I, yielding two internal *Pst*I fragments of 0.45 and 2.7 kb, respectively (Fig. 2). A double digestion of pNP5::Tn*1* (No. 105) DNA with both *Pst*I and *Hin*dIII allows us to establish the site of the Tn*1* insertion at 79.4% of the pNP5 map. The orientation of Tn*1* on pNP5 could be determined after digestion of pNP5::Tn*1* (No. 105) DNA with the enzyme *Bam*HI, which has unique cleavage sites both in Tn*1* and pNP5 (at 9.0%) (results not shown).

Previously, we have reported that the direction of transcription of the *uvrB* gene on pNP5 is anti-clockwise and mediated by a pMB9 promoter located between the *Eco*RI site (0%) and the *Hin*dIII site (4.5%) (Pannekoek et al., 1979). Combination of our data with those of Rubens et al. (1976), who identified polar and nonpolar orientations of Tn*1*, indicates that the Tn*1* insertion at 79.4% within the *uvrB* locus is oriented in the polar sense. In the following section evidence will be presented which indicates that the UV-sensitivity caused by Tn*1* (No. 105) is due to integration within the *uvrB* locus and not to the polar effect of Tn*1*.

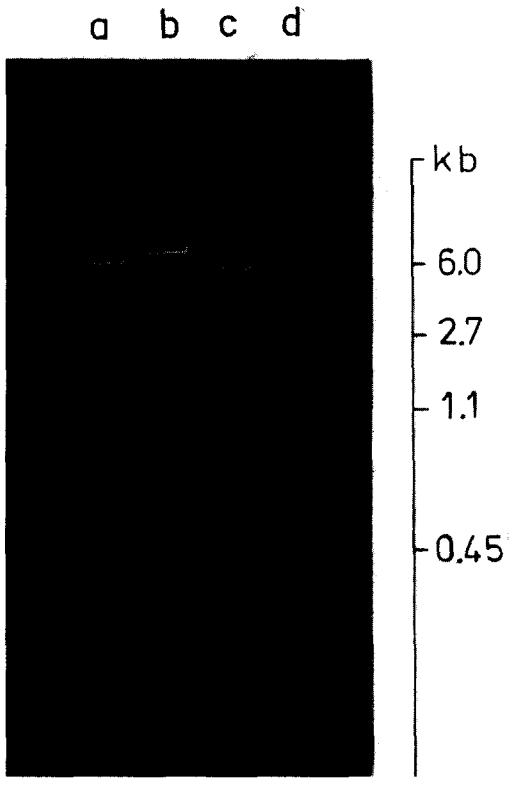

Fig. 2. Restriction endonuclease analysis of pNP5::Tn*1* DNAs. After enzymatic digestion DNA preparations were subjected to electrophoresis on a 0.8% agarose slab gel as described in MATERIALS AND METHODS. (a) pNP5::Tn*1* No. 104 digested with *Pst*I and *Hin*dIII. (b) pNP5::Tn*1* No. 105 digested with *Pst*I and *Hin*dIII. (c) pNP5::Tn*1* No. 107 digested with *Pst*I and *Hin*dIII. (d) Marker DNA fragments with lengths of, respectively, 6.82, 5.65, 4.77, 3.33 and 2.50 kb.

For a more detailed analysis of the *uvrB* region we have employed another transposon, Tn*5* (Km^r). Transposition of Tn*5* to plasmid pNP5 DNA was done by prolonged growth of an *E. coli* strain lysogenic for phage λ*b221rex*::Tn*5c*I857, which had been transformed with pNP5 DNA (Kreschmer and Cohen, 1977). A plasmid DNA preparation obtained from the resulting population of bacteria was used to transform strain HP3435(Δ*uvrB*); transformants were selected which exhibited a Tc^rKm^r phenotype. A large majority (634 out of 640) of independent Tc^rKm^r clones were Uv^r, analogous to integration of transposon Tn*1* into pNP5. Insertion of Tn*5* occurred mainly at two sites within the vector part of pNP5, namely at 34.1% (Nos. 502, 506) and at 65.0% (Nos. 503, 505; see Fig. 1). However, we also found six

Tc^rKm^r clones which were Uv^s; 5 out of 6 transformants had Tn*5* insertions at different sites within the cloned *uvrB* region (75.4%–100%). To analyse pNP5::Tn*5* DNA it is convenient to employ the restriction endonuclease *Bgl*I in combination with other enzymes, since *Bgl*I does not cleave the cloned *Eco*RI-fragment, but it cuts Tn*5* at several sites, e.g., in the inverted repeat (IR) flanking the transposon at approx. 60 bp from the junction between the transposon and bacterial DNA. A double-digestion of pNP5::Tn*5* with *Bgl*I and either *Eco*RI or *Bst*EII (Pannekoek et al., 1979) establishes the site of insertion of Tn*5*. An illustration of this procedure is given in Fig. 3, which shows an analysis of a DNA preparation of pNP5::Tn*5* (No. 501), demonstrating that the integration of Tn*5* had occurred close to the *Eco*RI site (100%) at approx. 150 bp. The positions of other Tn*5* insertions in the cloned *Eco*RI fragment are specified in Fig. 1. It can be observed that the five different sites of integration are scattered over the region between 75.4% and 100% which harbours the *uvrB* locus.

Due to the fact that Tn*5* is polar and mutagenic in both orientations (Berg, 1977, Kleckner, 1977), our data present no rigorous proof that the Tn*5* insertions are actually within the *uvrB* locus. It is, for instance, conceivable that the translation initiation codon of the *uvrB* gene is located anti-clockwise of Tn*5* insertion No. 501 and that UV sensitivity is caused by the polar effect exerted by Tn*5*. The following experiments should clarify this point.

(b) Elimination of polarity caused by Tn*5* insertions

Polarity due to nonsense mutations, such as in the IS-elements and transposons, is caused by transcriptional termination (Roberts, 1976; Das et al., 1976). The polar effect of IS*1* and IS*3* can be relieved by introduction of a mutation in the *suA* locus, which has been shown to encode the transcriptional termination factor rho (Malamy, 1970; Ratner, 1976). Evidence from in vitro experiments has indicated that IS*2* is polar in the presence, but nonpolar in the absence of rho (de Crombrugghe et al., 1973).

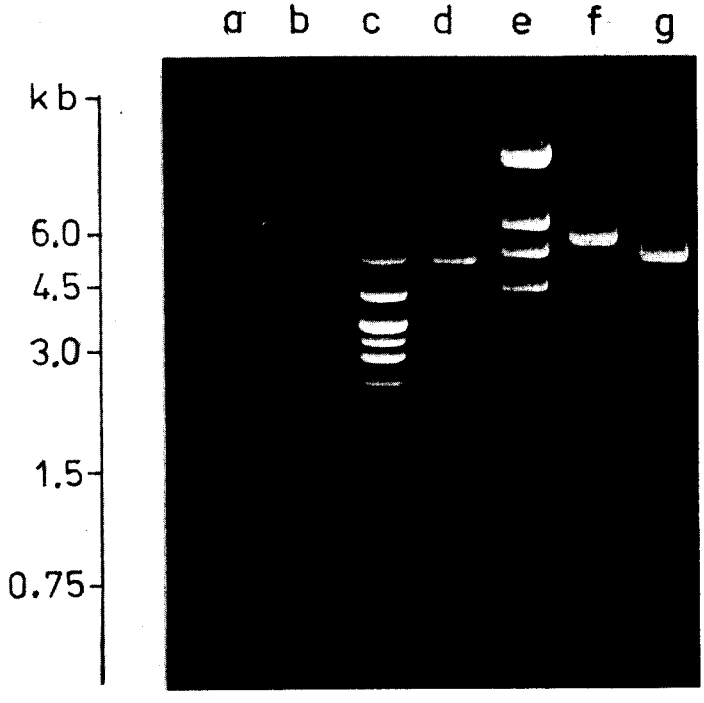

Fig. 3. Restriction endonuclease analysis of pNP5::Tn*5* No. 501 DNA. After enzymatic digestion DNA preparations were subjected to electrophoresis on an 0.8% agarose slab gel as described in MATERIALS AND METHODS. (a) pNP5::Tn*5* No. 501 digested with *Bgl*I and *Eco*RI. (b) pNP5::Tn*5* No. 501 digested with *Bgl*I and *Bst*EII. (c) Marker DNA fragments with lengths of, respectively, 5.30, 3.58, 3.26, 2.97, 2.42, 1.41, 1.11 and 0.79 kb (derived from plasmid pBR322 digested with various enzymes (Sutcliffe, 1978)). (d) pNP5::TN*5* No. 501 digested with *Bgl*I. (e) Marker DNA fragments of λ*b*2 DNA digested with *Eco*RI (20.8, 7.18, 5.65, 3.86 and 3.23 kb). (f) pNP5 digested with *Bgl*I. (g) pNP5 digested with *Bgl*I and *Hin*dIII.

Although the length and the restriction endonuclease patterns of IS*2* and the IRs of Tn*5* are similar, they are not homologous (Berg and Drummond, 1978; J. Hille, unpublished observation). Nevertheless, assuming that the polar effect of Tn*5* is due to some transcriptional termination sequence, we have attempted to relieve the polarity of Tn*5* by the introduction of a chromosomal *suA* mutation (Van de Putte et al., 1978). However, no alteration of the Uv^s phenotype of *uvrB* deletion strains carrying pNP5::Tn*5* (e.g. No. 501) was observed. This observation means that either all Tn*5* insertions had occurred within the *uvrB* gene or a mutation in the *suA* locus cannot relieve the polarity of Tn*5*.

Another way to relieve the polarity might have been the removal of a large part of the IRs. We have tested this hypothesis in the following way. Plasmid pNP5 has no restriction site for the enzyme *Xho*I, but Tn*5* contains three cleavage sites for *Xho*I, one in each IR and the third in the Km^r gene. Digestion of pNP5::Tn*5* DNA yields three fragments of 2.3, 2.4 and 8.2 kb, irrespective of the position of Tn*5* on the plasmid (Fig. 4; lanes a and c). The two smaller fragments are internal segments of Tn*5*, since double digestion with *Xho*I and *Eco*RI (no *Eco*RI site in Tn*5*) does not alter the size of these fragments (results not shown). Tn*5* is approx. 5.2 kb long, of which 2.9 kb (2 × 1.45 kb) are contributed by the IRs (Berg et al., 1975). From these data we can deduce that *Xho*I cleaves within each IR at a distance of about 230 bp from the junction of the transposon and pNP5 DNA.

When pNP5::Tn*5* DNA is digested with *Xho*I and subsequently religated under conditions of a low concentration of the digestion products (2 nM termini) then it is expected that predominantly monomers are

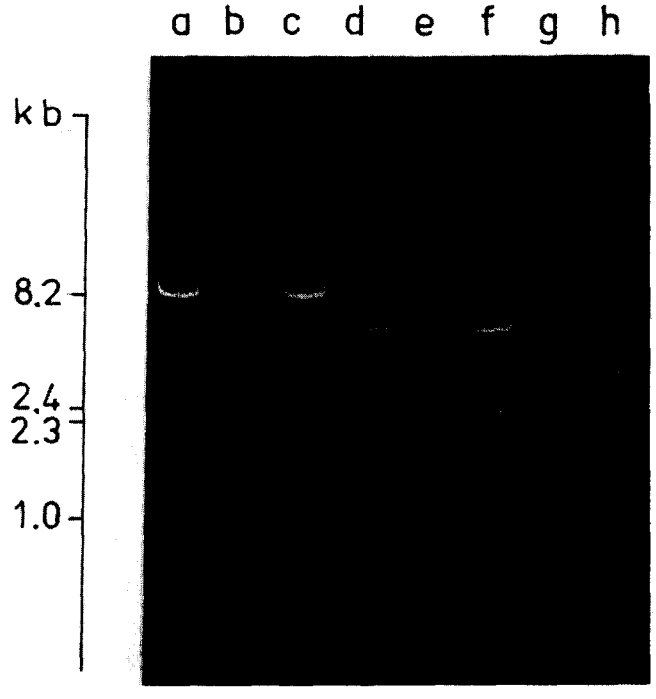

Fig. 4. Restriction endonuclease analysis of pNP5::Tn*5* DNA and its derivatives. After enzymatic digestion DNA preparations were subjected to electrophoresis on an 0.8% agarose slab gel as outlined in MATERIALS AND METHODS. (a) pNP5::Tn*5* No. 501 DNA digested with *Xho*I. (b) pNP5::Tn*5*-501Δ DNA digested with *Xho*I (no *Xho*I site; plasmid DNA preparations from 40 independent $Tc^rUv^rKm^s$ clones revealed the same pattern). (c) pNP5::Tn*5* No. 504 DNA digested with *Xho*I. (d) pNP5::Tn*5*-504Δ DNA digested with *Xho*I (no *Xho*I site; plasmid DNA preparations from 30 independent $Tc^rUv^sKm^s$ clones revealed the same pattern). (e) DNA from a deletion mutant of pNP5::Tn*5*-504Δ digested with *Eco*RI (fragment of 4.1 kb). (f) pNP5 DNA digested with *Eco*RI (fragments of 5.3 and 2.5 kb). (g) DNA from a deletion mutant of pNP5::Tn*5*-504Δ digested with *Bst*EII (fragment of 4.1 kb). (h) pNP5 DNA digested with *Eco*RI and *Bst*EII (fragments of 3.18, 2.12, 1.24 and 1.03 kb) (band of 0.22 kb is not visible in this experiment).

formed (Dugaiczyk et al., 1975; De Vries et al., 1976). Consequently, the *Xho*I fragments of 2.3 and 2.4 kb which originate from Tn*5* will be preferentially removed from the covalently closed replicons. The ligated mixture was used to transform strain HP3435(Δ*uvrB*) and transformants were selected for a Tc^r (Km^s) phenotype. Such clones were further analyzed for their resistance to UV-irradiation, which would be indicative for re-functioning of the cloned *uvrB* region. The results of this experiment, using pNP5::Tn*5* DNA (Nos. 508, 504, 507 and 501), are presented in Table I. In general, our data show that the Uv^s phenotype of the *uvrB* deletion strain harbouring either pNP5::Tn*5* Nos. 508, 504 or 507 does not change upon removal of a large part of the transposon. In contrast to these results, the data for pNP5::Tn*5* No. 501 reveal that almost all Tc^rKm^s clones are fully Uv^r. At this point we conclude that Tn*5* insertions 508, 504 and 507 have occurred within the *uvrB* locus, whereas Tn*5* insertion 501 is located ahead of the region essential for Uv^r. Apparently, the in vitro procedure, followed by transformation of the ligated digestion products relieves in most cases the polarity exerted by an intact Tn*5* insertion.

Further examination of the results with pNP5:: Tn*5* No. 504 shows that a few clones (2%) are found which display a Uv^r phenotype, in contrast to the majority of the clones, which remained Uv^s. Such exceptions were not detected with Tn*5* insertions 507 and 508, presumably since in these cases only 20–30 colonies were analysed. Conversely, digestion of pNP5::Tn*5* No. 501 DNA with *Xho*I, religation and subsequent transformation results in a few clones (2%) which remain Uv^s, whereas most of the Tc^rKm^s clones have a Uv^r phenotype. Apparently, the polarity of the Tn*5* insertion 501 is in a few exceptional cases not relieved. To search for an explanation of the observed heterogeneous phenotype of the resulting transformants we have isolated plasmid DNA from both $Tc^rKm^sUv^s$ and $Tc^rKm^sUv^r$ clones and analysed the structure of these DNA preparations with restriction enzymes. Plasmid DNA from pNP5::TN*5* No. 504 DNA digested with *Xho*I, religated and subsequently replicated in vivo is designated pNP5::Tn*5*-504Δ.

The expected length of pNP5::Tn*5*-504Δ DNA (or pNP::Tn*5*-501Δ) is 8.2 kb (12.9 – (2.3 + 2.4)). Furthermore, it is expected that pNP5::Tn*5*-504Δ DNA contains a unique *Xho*I site. However, every plasmid DNA preparation isolated from $Tc^rKm^sUv^s$ and from $Tc^rKm^sUv^r$ clones of either pNP5::Tn*5*-504Δ or pNP5::Tn*5*-501Δ DNA was devoid of an *Xho*I cleavage site (Fig. 4; lanes b and d). Moreover, the presence of an insertion of 0.46 kb, formed by the remaining segments of DNA from the IRs between

TABLE I

Elimination of the polarity exerted by transposon Tn*5*

Plasmid treated with *Xho*I and T4-DNA ligase	Position of Tn*5* on pNP5 map (%) [a]	Frequency of Uv^r among Tc^rKm^s bacteria [b]
pNP5::Tn*5* No. 508	77.7	0 per 20 (0%)
pNP5::Tn*5* No. 504	85.0	6 per 324 (2%)
pNP5::Tn*5* No. 507	91.8	0 per 30 (0%)
pNP5::Tn*5* No. 501	98.1	294 per 299 (98%)

[a] A schematic representation of plasmid pNP5 harbouring various transposons as well as restriction sites given in Fig. 1.
[b] Determination of Uv^r was done as described previously (Pannekoek et al., 1979).

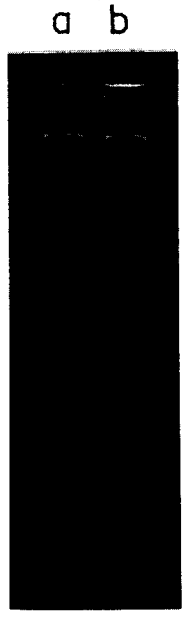

Fig. 5. Restriction endonuclease analysis of pNP5 DNA and pNP5::Tn*5*-504 DNA. After enzymatic digestion DNA preparations were subjected to electrophoresis on a 5% polyacrylamide slab gel as described in MATERIALS AND METHODS. (a) pNP5 DNA digested with *Hin*fI. (b) pNP5::Tn*5*-504Δ DNA digested with *Hin*fI. Identical restriction endonuclease patterns of these DNAs were also obtained upon digestion with *Alu*I or *Hae*III (results not shown).

the *Xho*I sites and the junctions, was not detected in the DNA; a comparison between the digestion patterns of pNP5::Tn*5*-504Δ DNAs, pNP5::Tn*5*-501Δ DNAs and pNP5 DNA with either *Eco*RI or *Eco*RI and *Bst*EII could not reveal any difference (results not shown). Even digestion of these DNAs with restriction endonucleases of a lower specificity, like *Hin*fI (*Alu*I, *Hae*III) did not show any difference in the structure of these DNAs (Fig. 5). Obviously, after propagation in bacteria the insertion of 0.46 kb was removed from the DNA. Based on a comparison between the restriction endonuclease patterns of pNP5 DNA and pNP5::Tn*5*-504Δ DNA (or pNP5:: Tn*5*-501Δ DNA) we conclude that the removal in vivo of the 0.46 kb insertion is fairly accurate, as judged by the observation that, except for one particular clone, in no case differences are detectable after digestion with various restriction enzymes.

The particular, quite different, clone which exhibited a $Tc^rKm^rUv^s$ phenotype contained plasmid DNA substantially smaller than pNP5 DNA. An analysis of this shortened DNA, given in Fig. 4 (lanes e, f, g, and h), indicates that a deletion was generated extending from the site of integration of Tn*5* insertion 504 up to almost the origin of replication. The resulting plasmid (4.1 kb; Tc^r) contains single sites for *Eco*RI and *Bst*EII, whereas pNP5 has two *Eco*RI and two *Bst*EII sites. Clearly, the *Bst*EII site (86.7%) located adjacent to the site of insertion 504 (85.0%) has been preserved. However, generation of "visible" deletions apparently is exceptional, since this is the sole case among 40 plasmid DNA preparations isolated from different Tc^rKm^s clones, obtained after removal of Tn*5*.

If the in vivo removal of the remaining 0.46 kb insertion is precise, then the full activity of the *uvrB* locus should be restored. Imprecise removal, i.e. a remaining insertion of a few basepairs or a deletion of a few basepairs, most likely will not yield a functional *uvrB* locus. Hence, the appearance of Tc^rUv^r and Tc^rUv^s clones will reflect, respectively, precise and imprecise removal of the 0.46 kb insertion. In the case of pNP5::Tn*5*-504Δ DNA, originating from a Tn*5* insertion located probably in the middle of the *uvrB* gene (Pannekoek et al., 1980), six colonies of the 324 Tc^rKm^s clones were Uv^r. From these data we conclude that the ratio of imprecise vs. precise removal is about 50 to 1. Again, it must be emphasized that the restriction enzyme patterns of pNP5:: Tn*5*-504Δ DNA and of pNP5::Tn*5*-501Δ DNA with various enzymes were identical, irrespective of whether DNA was isolated from Uv^s or Uv^r clones. A possible explanation for the in vivo removal of the 0.46 kb insertion will be outlined in the DISCUSSION.

We assume that the predominant imprecise removal of the insertion from pNP5::Tn*5*-504Δ DNA occurs also when Tn*5* is located at another position. Hence, imprecise removal of the insertion from pNP5::Tn*5*-501Δ DNA does not prevent the restoration of the activity of the *uvrB* locus. Thus, although a site-specific mutation (a small insertion or deletion) will probably remain in the manipulated pNP5:: Tn*5*-501Δ DNA, it does not interfere with full expression of the *uvrB* locus. The reactivation of the *uvrB* locus, previously mutated by the Tn*5* insertion 501, can be interpreted in two ways. (i) Transposon Tn*5* No. 501 had been integrated in a promoter-proximal DNA segment which precedes the *uvrB* locus; this hypothetical segment would not have a function in determining Uv^r. The Uv^s phenotype of strain HP3435(Δ*uvrB*) carrying pNP5::Tn*5* No. 501 will then be caused by polarity. (ii) Alternatively, the segment of DNA between the integration site of Tn*5* insertion 501 and the genuine *uvrB* promoter belongs to the *uvrB* locus, but the amino-acid sequence encoded by this segment has no significance for Uv^r. Processing of precursor protein molecules would be compatible with this possibility.

We have further concluded that Tn*5* insertions 507, 504 and 508 are actually within the *uvrB* locus. In a previous paragraph we have described a strain containing pNP5 DNA carrying transposon Tn*1* (No. 105) in the polar orientation. Tn*1* insertion 105 is located between Tn*5* insertions 504 and 508. Consequently, we conclude that Tn*1* insertion 105 is, similar to Tn*5* insertions 504, 507 and 508, also located within the *uvrB* locus.

Furthermore, we can deduce from the data presented so far that the region within the cloned *Eco*RI fragment which is essential for Uv^r extends approximately from 75.4% of the pNP5 map to 98.1% (insertion site of Tn*5* insertion 501) and spans about 1.8 kb (see Fig. 1). Moreover, our data show that treatment of pNP5::Tn*5* DNA with *Xho*I and T4-DNA ligase, followed by culturing transformed bacteria can efficiently relieve the polarity exerted by Tn*5*. In the DISCUSSION it will be outlined that this

procedure can be applied more generally for the investigation of cloned complex genetic entities.

DISCUSSION

(a) Functional and structural aspects of the *uvrB* locus

Plasmid pNP5 lacks the *uvrB* promoter, but contains the genetic information to fully complement the defects due to chromosomal *uvrB* deletions, which extend from the *bioB* gene to the *chlA* locus (Pannekoek et al., 1978). The maximal protein coding capacity of the cloned region (1920 bp) on pNP5 DNA corresponds with a polypeptide of about 70 000 daltons. Furthermore, we have mentioned before that pNP5 DNA specifically encodes, in minicells, a protein with M_r of 75 000; this protein is a fusion product initiated on the pMB9 part of pNP5 (Pannekoek et al., 1980). Apparently, this function product substitutes for the genuine *uvrB*-coded protein in the establishment of a Uv^r phenotype.

This assumption is compatible with the results presented in this paper, concerning the properties of plasmid pNP5::Tn*5* No. 501 (site of integration at 98.1% of pNP5; see Fig. 1). Relief of polarity exerted by Tn*5* insertion 501 restores the Uv^r phenotype indicating that the segment of DNA (about 150 bp) located between the *Eco*RI site (100%) and Tn*5* insertion 501 (98.1%) can be altered without affecting the Uv^r phenotype. Two explanations can be advanced to account for this observation. (i) The promoter-proximal segment does not belong to the *uvrB* gene and has no function in determining a Uv^r phenotype. (ii) This segment belongs to the *uvrB* gene, but it can be omitted without loss of Uv^r.

Recently, we have cloned the entire *uvrB* locus including its transcriptional regulatory elements (H. Pannekoek et al., submitted). It was shown that the *uvrB* locus consists of a single gene that encodes a protein of about 80 000 daltons. Consequently, we favour the second explanation outlined in the previous paragraph. Hence, we propose that only the carboxy-terminal part of the *uvrB* gene product is essential for Uv^r, whereas a portion of the amino-terminal part of the protein can be omitted. A similar partitioning of a function within a polypeptide chain has been observed before; e.g. splitting of the enzyme DNA polymerase I by an in vitro treatment with a proteolytic enzyme yields a protein fragment which has retained the full polymerising activity displayed by an intact protein preparation (Klenow et al., 1971).

(b) In vivo elimination of peculiar DNA structures

The 0.46 kb insertion, which remains in pNP5::Tn*5* DNA after digestion with *Xho*I and subsequent in vitro religation, has a remarkable composition. It consists of two adjacent DNA sequences that are identical but in an inverted orientation. In contrast with the structure of most transposons, in this particular case the two inverted repeats (about 230 bp each) do not enclose a segment of heterologous DNA. Possibly such structures are deleterious for the plasmid DNA replication and as a result viable colonies (resistant to tetracycline) are selected that have lost the insertion. We envisage two modes of removal of the remnants of the IRs from pNP5 DNA: (i) By a direct excision mechanism; this process apparently would be rather inaccurate, since the results with pNP5::Tn*5* No. 504 DNA (Table I) suggest that predominantly imprecise excision occurs. (ii) By "slippage" of DNA replication caused by the unusual structure of the insertion, composed of two directly adjacent inverted repeats. "Slippage" of DNA replication might yield plasmid DNA molecules that differ from pNP5 DNA by only a few bp.

Similar results as reported here have been obtained by Sadler et al. (1980). These authors have cloned multiple adjacent copies of a 40 bp fragment harbouring the *E. coli* lactose (*lac*) operator into the unique *Eco*RI site of plasmid pMB9 (Rodriguez et al., 1976). They observed that in vivo stability of such recombinant plasmids is associated with direct repeat orientation of the *lac* operators. Inverted repeat orientations of pairs of operators lead to rapid reduction, in vivo, of the number of cloned operators. We propose that neither the length of adjacent inverted repeats nor the DNA sequence is essential for an in vivo elimination.

(c) Other applications of elimination of polarity exerted by transposons

A prerequisite for a more general application of the procedure to eliminate polarity associated with transposons is the presence of restriction endo-

nuclease sites within inverted or direct repeats of the transposon, but not within the cloned DNA fragment. Using Tn*5* one may choose between the enzymes *Xho*I, *Hin*dIII and *Bgl*I to eliminate most of the transposon and simultaneously preserve the cloned fragment. Suitable restriction sites in the IS-elements characterized so far, have been listed by Szybalski (1977). Interestingly, Tn*9* (Cm^r) is flanked by direct repeats of IS*1* (MacHattie and Jackowski, 1977). Since IS*1* has unique *Pst*I and *Bal*I sites, elimination of Tn*9* with *Pst*I (*Bal*I) will yield a single site-specific IS*1* copy; the latter can be suppressed by a *suA* mutation (Malamy, 1970). Experiments are in progress to obtain evidence for this presumption. Furthermore, restriction endonuclease patterns have also been established for the IRs of Tn*501* (Hg^{2+}-resistance). These IRs contain an *Eco*RI site near the junction of the transposon and bacterial DNA (Bennett et al., 1978). Thus, provided a cloned fragment does not contain *Eco*RI sites then digestion of the DNA carrying Tn*501* with this enzyme may relieve polarity as well.

In conclusion, we have mapped on a recombinant plasmid pNP5, which encodes Uv^r in *uvrB* deletion strains, the region essential for this phenotype, using insertional inactivation with transposons. In addition, a procedure is described that causes relief of the polarity exerted by a transposon. The method employed can be extended to other transposons integrated in complex genetic units.

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